Non-pathogenic aquatic bacteria activate the immune system and increase predation risk in damselfly larvae

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Summary

1. Pathogens can increase vulnerability to predation through their harmful effects on hosts. Recently it was shown that the mere activation of the immune system by pathogens may increase the host's risk of predation. Here we test whether exposure to non-pathogenic bacteria also activates the immune system and thereby increases vulnerability to predation.
2. We exposed *Enallagma cyathigerum* damselfly larvae to a non-pathogenic strain of the bacterium *Escherichia coli* and measured immune defence, anti-predator behaviour and survival times in the presence of larval dragonfly predators. To evaluate whether non-pathogenic bacteria also generated energy-based trade-offs leading to other fitness costs, we also quantified growth rate and survival in the absence of predators.
3. Exposure to the non-pathogenic bacterium did not affect survival in the absence of the predator but increased growth rate, possibly a response to reduce exposure time to the bacterium. Larvae exposed to the bacterium activated their immune response as shown by an increase in the activity of phenoloxidase and the number of haemocytes. The bacterium affected anti-predator traits involved in avoiding detection by predators as well as traits involved in escape after detection. Pre-exposed larvae showed higher activity levels and further increased the number of feeding strikes in the presence of predation risk, possibly driven by energetic constraints. Pre-exposed larvae swam less often when attacked, but faster. This impaired anti-predator response came at the ecological cost of increased vulnerability to predation.
4. Our study demonstrated that exposure to non-pathogenic bacteria increases vulnerability to predation, which is a novel type of antagonistic interaction. This highlights the unexplored possibility that non-pathogens may play a role in maintaining variation in immune defence through insidious effects on predator-prey interactions. Since non-

pathogenic bacteria can be very abundant, this unexplored ecological cost of immune system activation in terms of increased predation may have major consequences in natural systems and may provide an unexplored new force underlying variation in immune defence.

Introduction

Organisms are embedded in complex biotic networks where they face opposing selective forces where exposure to one natural enemy, such as parasites and pathogens, may make them more vulnerable to another enemy, such as predators (Decaestecker, De Meester & Ebert, 2002; Hatcher *et al.*, 2006; Edeline *et al.*, 2008; Yin *et al.*, 2012). There is increasing attention for antagonistic ecological interactions with parasites and pathogens as these may contribute to the selective forces shaping and maintaining variation in immune defence, a central topic of ecological and evolutionary immunology (Rolff & Siva-Jothy, 2003; Schmid-Hempel, 2005). Several studies have demonstrated that parasites and pathogens may make hosts more vulnerable to predation as a result of harmful effects of the pathogen or parasite itself, including behavioural manipulation of the host by the parasite (Cézilly *et al.*, 2010; but see Perrot-Minnot *et al.*, 2012). Only recently it was documented that increased predation risk can be driven by the activation of the immune system itself whereby artificially immune-challenged animals increase foraging activity in order to meet the higher energetic demands and decrease responsiveness to predators (Rantala, Honkavaara & Suhonen, 2010; Otti *et al.*, 2012).

Interactions with non-pathogenic bacteria have been ignored in the context of trade-offs underlying the maintenance of variation in immune function (Sheldon & Verhulst, 1996; Schmid-Hempel, 2005). Yet, it has recently been documented that exposure to non-pathogenic bacteria can activate the immune system (Freitak *et al.*, 2007; Freitak, Heckel & Vogel, 2009; Willer, Müller & Bumann, 2012). We therefore hypothesize that non-pathogenic bacteria may also increase vulnerability to predation, hence may play a role in the maintenance of variation in immune defence. This could have major consequences in natural aquatic systems where non-pathogenic bacteria can be very abundant (Leff & Lemke, 1998).

We here test whether exposure to non-pathogenic bacteria activates the immune system in *Enallagma cyathigerum* damselfly larvae and thereby increases their vulnerability to predation. Damselfly larvae are important intermediate predators in aquatic food webs that are prey to large dragonfly larvae (Stoks, De Block & McPeek, 2005a). To mechanistically explain the predicted ecological cost of a non-pathogen induced immune activation, we scored the key behavioural anti-predator traits in damselfly larvae (Stoks, McPeek & Mitchell, 2003; Stoks *et al.*, 2005b; Strobbe *et al.*, 2009). To evaluate whether non-pathogenic bacteria also generated energy-based trade-offs leading to other fitness costs we also quantified growth rate and survival in the absence of predators.

Methods

Collecting and housing

Fifteen mated females of the damselfly *Enallagma cyathigerum* were collected in De Ruiterskuilen, a fishless pond in Opglabbeek (Belgium) containing larvae of the large dragonfly *Anax imperator* as the most important predator. Females were transferred to the laboratory to lay eggs. Ten days after hatching, larvae were placed individually in 200 ml cups. Throughout their life, larvae were reared under standard conditions of light (14:10 L:D), temperature (20 °C) and food (ad libitum *Artemia* nauplii five days a week [average daily dose = 1347, SE = 102, N = 15]). When larvae moulted into the final instar, they were used for the experimental trials. Starting from that point the larvae were fed seven days a week with *Artemia* nauplii.

Experimental setup

Sixty larvae were exposed to each of two bacterium treatments (absent versus present) for seven days (total of 120 larvae). During the exposure period, larvae were placed individually

in glass vials (100 ml) filled with 50 ml synthetic pond water (for details see Janssens & Stoks 2013) with daily refreshment of the medium to avoid increases in bacterial concentration.

For the bacterial exposure we used the non-pathogenic ATCC 11775 *E. coli* strain (Guerrero-Beltran & Barbosa-Canovas, 2005). This strain was genetically modified by insertion of a plasmid coding for *dsRed* fluorochrome and kanamycin resistance, allowing it to grow on a culture medium containing kanamycin. Daily throughout the experiment, we prepared an *E. coli* solution with a concentration of 1×10^9 CFU 100 ml^{-1} in mili-Q water using a spectrophotometer. We added 1 ml of this solution to vials of the bacterial treatment resulting in a concentration of 2×10^7 CFU 100 ml^{-1}. To the control treatment we added 1 ml of mili-Q water. We chose this concentration as we have previously shown reduced growth rates in the study species at this concentration (Janssens & Stoks, 2013) and because this is a concentration of non-pathogenic bacteria regularly encountered in water bodies. This concentration of *E. coli* is in the upper range of concentrations for this particular bacterial species reported in field studies (e.g., Kulkoyluoglu *et al.*, 2007), including Flemish surface waters (Aquafin, 2012). Total bacterial concentrations reported in natural unpolluted aquatic systems, however, regularly exceed 10^9 CFU 100 ml^{-1} (Kirschner & Velimirov, 1997; Palijan, 2012). As the large majority of natural aquatic bacterial communities consist of non-pathogenic bacteria, it is likely that damselfly larvae will be frequently exposed to the concentrations of non-pathogenic bacteria we used.

Response variables

Because all response variables could not be measured on the same larva, we worked with two sets of 30 larvae per treatment combination (total of 120 larvae). On a first set of 30 larvae per treatment combination we studied growth rate, activity, anti-predator behaviour and immune defence. We quantified growth rate across the 7-day exposure period as [ln(final

mass) – ln(initial mass)] / 7 days (Stoks *et al.*, 2005a) by weighing each larva to the nearest 0.01 mg at the beginning and end of this period.

Larval activity was scored immediately after the 7-day exposure period in the absence of the bacterium based on the protocol by Janssens & Stoks (2012). We will refer to the larvae that had previously been exposed to bacteria during the exposure period as the pre-exposed larvae. An important strategy of *Enallagma* larvae to avoid detection by predators is to reduce their activity (Stoks *et al.*, 2005c; Janssens & Stoks, 2012). Each larva was individually placed in a 2 L container filled with 250 ml synthetic pond water. After an acclimation period of seven minutes we added 2 ml of *Artemia* nauplii solution (ca. 2000 nauplii) to the container. Thereafter, we recorded the number of feeding strikes (food intake) and walks for seven minutes. Afterwards, the larva was transferred to an identical container filled with 250 ml medium to which 1 ml of predator medium was added to impose predation risk. The larva was again acclimated for seven minutes, after which we added the same number of *Artemia* nauplii and recorded the number of feeding strikes and walks for seven minutes. We observed larval activity without predator cues first to avoid any lag effect of predator cues during the second observation period. *Enallagma* damselfly larvae do not show food saturation during such a second observation period (Janssens & Stoks, 2012). Therefore, any difference in larval activity levels between the two observation periods can be attributed to predation risk. Predator medium was prepared by homogenizing two conspecific damselfly larvae in 20 ml of water where a large *Anax* dragonfly predator, an important predator of *Enallagma* (Stoks *et al.*, 2005a), had eaten a conspecific larva. This way, the larvae received a natural cocktail of predator kairomones and alarm stimuli, chemical predator cues to which *Enallagma* larvae are known to react with anti-predator behaviour (Mortensen & Richardson, 2008; Janssens & Stoks, 2012).

Directly after the activity test, swimming performance was measured based on the protocol of Strobbe *et al.* (2009). Note that by first testing each larva in the activity test, we ensured that each larva had been exposed to predator cues before it responded to the simulated predator attack, thereby mimicking the situation in nature. Moreover, first performing the swimming test and then the activity test might have generated lag effects of predation risk (simulated predator attack) thereby precluding the quantification of baseline activity levels in the absence of predation risk. Swimming away is the main escape strategy of *Enallagma* damselfly larvae when attacked by large dragonfly larvae (Stoks & McPeek, 2006). Each larva was placed in a large container filled with 250 ml synthetic water without the *E. coli* bacterium and allowed to acclimate for ten minutes. Thereafter, we stimulated the larva to swim by tapping on its abdomen with a plastic pipette. This was done five times. The swimming propensity was defined as the number of times the larva swam (ranging from zero to five). Three swimming bouts per larva were filmed with a digital camera (30 Hz) (Basler, AG, Ahrensburg, Germany) connected to a computer using Streampix software (Norpix, Inc., Montreal, Canada). Larvae that swam fewer than three times when testing their swimming propensity were stimulated further until three swimming bouts were obtained. We quantified the swimming speed per swimming bout using Image Pro Plus v5 (Media Cybernetics, Inc., Bethesda, MD, USA). Swimming speed (cm s^{-1}) was calculated as the distance the larvae covered during the first 15 frames of one swimming bout divided by the duration (0.5 seconds). We chose to digitize the first 0.5 s to have enough frames to accurately calculate swimming speed and given that this is probably the most critical period for damselfly larvae to escape attacks from sit-and-wait predators such as dragonfly larvae that do not chase their prey after initial attack (Dayton *et al.*, 2005). We averaged the speed per larva of the three swimming bouts for later analysis.

Subsequently animals were stored at -80°C to quantify two key components of an insect's immune system (Siva-Jothy *et al.*, 2005; Gonzalez-Santoyo & Cordoba-Aguilar, 2012): the number of haemocytes and the activity of phenoloxidase. To quantify the number of haemocytes, we used the protocol of Campero *et al.* (2008). In the first step, we extracted the haemolymph by perfusing the body of the larvae with 300 µl phosphate buffer (PBS, 50 mM, pH 7.4). We then placed 20 µl of the haemolymph sample into a well of a multiwell microscope slide, stained it with 5 µl ethidium bromide (2.5 mM) and placed the slide at 4°C in the dark for 2 hours. Afterwards, we took five pictures from each well using a fluorescence microscope and the number of haemocytes in each picture was counted. The sum of these five counts per larva was used for statistical analysis. Immediately after extracting the haemolymph, the larvae were homogenized in the phosphate buffer, centrifuged for 5 minutes (13,200 rpm, 4°C) and kept on ice. The phenoloxidase activity was quantified using a modified version of the protocol of Stoks *et al.* (2006). A 96 well microtiter plate was filled with 55 µl of phosphate buffer and 20 µl of the body supernatant. Then, we added 5 µl of chymotrypsin (5 mg ml^{-1} mili-Q water) and incubated the mixture for 5 minutes at room temperature. This way all pro-enzyme prophenoloxidase present was converted into phenoloxidase. As a final step, we added 120 µl L-DOPA (10 mM in PBS). We measured absorbance at 490 nm for 30 minutes every 20 seconds at 30 °C. Phenoloxidase activity was determined as the slope of the linear part (500-1500 s) of the reaction curve. Measurements were run in duplicate per larva and the means were used for statistical analysis.

On a second set of 30 larvae per treatment combination we tested after an identical 7-day exposure period whether the observed effects of bacterial exposure on anti-predator behaviour and escape swimming speed (see results) resulted in increased vulnerability to predation. For this, we tested survival times in the presence of a larval dragonfly predator (based on Janssens & Stoks, 2012). The larva was placed in a container filled with 150 ml

synthetic water without the bacterium and allowed to acclimate for ten minutes. Afterwards, one *Anax imperator* predator was added to the container and the time that the predator needed to catch the damselfly larva was recorded. Survival times longer than 120 minutes were considered right-censored.

Statistical analyses

All analyses were run in STATISTICA v11 (StatSoft 2011, Tulsa, OK, USA). Effects of bacterial exposure on survival during the exposure period were analyzed using a loglinear model. Effects of bacterial exposure on growth rate, escape swimming speed, phenoloxidase activity and number of haemocytes were analyzed using AN(C)OVAs. We initially included body mass as a covariate for swimming speed, but since it was not significant, it was removed. We included protein content and final body mass as covariates when analyzing phenoloxidase activity.

We used repeated-measures ANCOVAs to test for effects of bacterial exposure and predation risk on the number of walks and feeding strikes. These variables were first log-transformed to meet the assumption of normality. The bacterial exposure treatment was the independent variable and the activity of each larva successively measured with and without chemical predator stimuli were the repeats. Because larger larvae were more active, we added body mass as a covariate. When the test indicated a significant interaction between the bacterial exposure and the chemical predator stimuli, we performed linear contrasts to investigate the effect of the predator stimuli in the absence and presence of the bacterium.

To test for effects of bacterial exposure on swimming propensity, we used the Mann-Whitney U-test because the dependent variable was not normally distributed. Effects of previous bacterial exposure on survival times in the presence of the predator were tested with a survival analysis that allows right-censored data to be taken into account (i.e. larvae that

survived up to 120 minutes). We compared the survival curves between the two bacterium treatments using a Gehan's Wilcoxon test, the preferred, most powerful test when comparing two groups with many right-censored data (Fox, 2001). We also used a Cox regression to test whether larval size affected survival times.

Results

Throughout the 7-day exposure period survival was high (control: 95.4%, with bacterium: 92.4%) and unaffected by bacterial treatment ($\chi^2_1 = 0.45$, $P = 0.50$). Bacterial exposure had a positive effect on growth rate ($F_{1, 58} = 7.09$, $P = 0.010$; Fig. 1a). Bacterial exposure activated the immune system: both phenoloxidase activity ($F_{1, 56} = 4.91$, $P = 0.031$) and the number of haemocytes ($F_{1, 58} = 17.28$, $P < 0.001$) were higher in larvae exposed to the bacteria (Figs 1b-c).

The activity test showed that the bacterium affected anti-predator traits involved in avoiding detection by predators. Pre-exposed larvae showed higher walking activity and more feeding strikes in the absence of predation risk (Table 1, Fig. 2). The significant predation risk × bacterium interactions for walking activity and feeding strikes (Table 1) indicates different responses to predation between control larvae and pre-exposed larvae. Control larvae reduced walking activity (contract analysis, control larvae without predator cues vs control larvae with predator cues, $P = 0.011$) and feeding strikes ($P < 0.001$) in the presence of predation risk. Pre-exposed larvae did not reduce walking activity (contrast analysis, pre-exposed larvae without predator cues vs pre-exposed larvae with predator cues, $P = 0.32$) and increased feeding strikes in the presence of predation risk ($P = 0.029$) (Fig. 2).

The swimming test indicated that previous bacterial exposure shaped the escape swimming response of the larvae. Pre-exposed larvae showed a lower swimming propensity

than larvae not pre-exposed to the bacteria ($U = 103$, $N_1 = 30$, $N_2 = 30$, $P < 0.001$). Furthermore, pre-exposed larvae showed a higher swimming speed ($F_{1, 58} = 12.37$, $P < 0.001$; Fig. 3).

The results of the survival test indicated that pre-exposed larvae had significantly shorter survival times when placed together with the dragonfly predator (Gehan Wilcoxon test statistic = -2.18, $P = 0.029$) (Fig. 4). Larval size had no effect on survival time (Cox regression, $\chi^2 = 0.92$, df = 1, $P = 0.33$, slope = -0.033 (SE = 0.034), hazard ratio = 0.97).

Discussion

Exposure to non-pathogenic bacteria activated two key components of the insect's immune system (Siva-Jothy *et al.*, 2005; Gonzalez-Santoyo & Cordoba-Aguilar, 2012): both phenoloxidase activity and the number of haemocytes increased. We consider it unlikely that this upregulation is a response to a novel bacterial species. Although we do not have direct evidence for the presence of *E. coli* at the collection site, given its widespread distribution (Espinoza-Urgel & Kolter, 1998) it is very unlikely to be absent in the particular water body where the studied damselfly species occurs. Being flying insects, moreover, damselflies show high levels of gene flow among water bodies (e.g., Johansson *et al.*, 2013), making it unlikely that the *E. cyathigerum* genotypes present at a given water body would never have encountered *E. coli* in other water bodies. Furthermore, an immune response to familiar non-pathogenic bacteria has been observed before and interpreted as immune priming of the host (Freitak *et al.*, 2007, 2009) or the inability to distinguish between pathogenic and non-pathogenic bacterial challenges (Willer *et al.*, 2012).

Upregulation of these immune parameters is known to be costly (e.g. Siva-Jothy & Thompson, 2002; De Block & Stoks, 2008a). Nevertheless, in contrast with other studies

(e.g., Moret & Schmid-Hempel, 2000; Jacot, Scheuber & Brinkhof, 2004; Freitak *et al.*, 2007), immune activation in the current study was not traded off against the measured life history traits: survival in the absence of predators was not affected and growth rate increased in immune-activated animals. Similarly, exposure to non-pathogenic *E. coli* was not lethal and accelerated larval development in *Tribolium castaneum* beetles (Roth & Kurtz, 2008). Such life history acceleration probably reflects plastic life history adjustments of the host upon sensing a potentially pathogenic environment, which may be adaptive by shortening exposure during the more vulnerable larval stage (Roth & Kurtz, 2008). The absence of a trade-off between immune function and these life history traits in the current study may be explained by ad libitum food conditions and the higher food intake. Trade-offs, including those with immune traits, can often only be detected under food-limited conditions (e.g. Valtonen *et al.*, 2010) and may be masked when animals increase their energy content (Van Noordwijk & de Jong, 1986). It should be noted that while upregulation of the immune function did not result in a growth cost, and instead resulted in a growth increase, this growth increase itself is likely to be costly. Apart from the fact that it may have contributed to increased vulnerability to predation through increasing activity levels, other potential costs of this increased growth rate in pre-exposed larvae are a lower investment in defence mechanisms such as stress proteins (e.g., Stoks & De Block, 2011), lower energy reserves (e.g., Stoks *et al.*, 2006), the accumulation of oxidative damage (e.g., De Block & Stoks, 2008b) and reduced fecundity (e.g., Dmitriew, 2011).

Higher activity levels together with the impairment of anti-predator responses in pre-exposed larvae, and this despite an increase in escape swimming speed, seemed to result in increased vulnerability to predation. Instead of typical activity reductions in the presence of predation risk in *Enallagma* larvae (Stoks *et al.*, 2003; Stoks *et al.*, 2005c), pre-exposed larvae did not reduce walking and increased foraging activity under predation risk, thereby

increasing their visibility to the visually oriented dragonfly predators. Note that this pattern is unlikely to be driven by a saturation effect after the first 7-min observation period as we have shown before that *E. cyathigerum* larvae can maintain much higher foraging rates for 2 hours (Janssens & Stoks, 2013). Moreover, any saturation would make the increase in food intake during the second observation period (hence in the presence of predator cues) in pre-exposed larvae conservative. The absence of a lowering of activity levels to avoid predation, and this despite already high baseline levels, in pre-exposed larvae may reflect the increased energetic needs associated with the costly investments in immune defence and in increased growth rates. Moreover, once attacked, larvae showed a lower propensity to swim away which has been associated with an increased probability of being killed by dragonfly predators (McPeek, Schrot & Brown, 1996; Strobbe *et al.*, 2009). Although we cannot fully exclude any order effects due to first running the activity test and then the swimming test, we see no reason to think this may have affected the differences between pre-exposed and non pre-exposed larvae. Moreover, the used order best mimics the natural sequence of predator-prey encounters in nature. The observed differences in activity and swimming behaviour between both larval groups are consistent with the independent results of the survival experiment, which was performed with animals whose activity and swimming were not tested, hence on larvae that could not have been affected by any potential order effects. Note that any size differences between control larvae and the faster growing pre-exposed larvae cannot explain the differences in survival times indicated by the Cox regression. This is consistent with previous studies that could not detect any size-selective predation of large larval dragonfly predators on damselfly larvae (e.g., Brodin & Johansson, 2004; Strobbe *et al.*, 2009).

In nature, predators, and not just prey, would be likely to be exposed to the bacteria. An interesting future addition to our experiment would therefore be to also pre-expose dragonfly predators to the bacteria. To the best of our knowledge, no studies have looked at

effects of an elevated immune system upon predation efficacy. As damselfly larvae are predators themselves (in our study on *Artemia*), our results indicate that pre-exposed predators probably increase predation rates. While this awaits experimental testing for predators of damselfly larvae, if confirmed this would suggest that current findings of an increased vulnerability to predation in pre-exposed prey may be magnified in the presence of pre-exposed predators.

Empirical proof for immunity-mediated costs of predation is rare. Two previous studies documented behavioural changes that increased vulnerability to predation after activation of the immune system. Immune-challenged adult male *Calopteryx* damselflies reduced territory holding potential in association with the activation of the immune system and this combination led to higher predation rates by birds (Rantala *et al.*, 2010). The underlying mechanism was unclear, but one potential mechanism suggested by the authors was that immune-challenged males had a poorer physical condition resulting in a reduced ability to escape a predatory attack. Immune-challenged male *Gryllus campestris* crickets spent more time outside their burrows and reacted more slowly to a simulated predator attack and this caused higher predation rates by shrews (Otti *et al.*, 2012). Interestingly, in the latter study it was also shown that while the propensity to escape was lowered, the escape speed was not impaired. This may reflect similar effects of immune activation on energy depletion or on neurotransmission in crickets and damselfly larvae.

An important contrast between our study and the two previous studies that showed that immune activation increased predation risk is that these studies used artificial mechanical wounding. The *Calopteryx* damselfly males were immune-challenged by inserting a nylon filament into the abdomen (Rantala *et al.*, 2010) (mimicking the mouthparts of ectoparasitic water mites, Nagel *et al.*, 2011). The *Gryllus* crickets were challenged by injecting lipopolysaccharides of pathogenic bacteria (Otti *et al.*, 2012). Such approaches, however,

neglect the main routes of exposure to parasites and pathogens (Freitak *et al.*, 2007). Moreover, wounding, even when not showing an effect in manipulated animals, may interact synergistically with the immune challenge. This was avoided in the current study by administering the bacterium through the water. More importantly, we have documented that exposure to living non-pathogenic bacteria has the potential to increase predation risk.

Our study shows for the first time that exposure to living non-pathogenic bacteria increases vulnerability to predation, which is a novel type of antagonistic interaction. This highlights the unexplored possibility that non-pathogens may play a role in maintaining variation in immune function through insidious effects on predator-prey interactions. Given that non-pathogenic bacteria can be very abundant, they may constitute an important selective factor in the context of trade-offs underlying the maintenance of variation in immune function (Sheldon & Verhulst, 1996; Schmid-Hempel, 2005). The central paradigm in ecological immunology is the presence of energy-mediated trade-offs between the benefits of activating the immune system in terms of defence against pathogens and parasites and the costs due reduced energy availability to other fitness-related functions (Sheldon & Verhulst, 1996; Schmid-Hempel, 2005). Costs of activating an immune response have been mainly focused on life history traits such a reduced survival (Moret & Schmid-Hempel, 2000). Together with two other recent studies (Rantala *et al.*, 2010; Otti *et al.*, 2012), our results underscore the potential of ecological costs in terms of increased vulnerability to predation to also play a role in maintaining variation in immune defence and extend this concept toward a neglected type of interacting species: non-pathogenic bacteria.

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References

Aquafin (2012) Uitvoering zwemwaterrichtlijn vermindert kustvervuiling. *Aqua*, **1**, 10-12.

Brodin T. & Johansson F. (2004) Conflicting selection pressures on the growth/predation risk trade-off in a damselfly. *Ecology*, **85**, 2927-2932.

Campero M., De Block M., Ollevier F. & Stoks R. (2008) Correcting the short-term effect of food deprivation in a damselfly: mechanisms and costs. *Journal of Animal Ecology*, **77**, 66-73.

Cézilly F., Thomas F., Medoc V. & Perrot-Minnot M.J. (2010) Host-manipulation by parasites with complex life cycles: adaptive or not? *Trends in Parasitology*, **26**, 311-317.

Dayton G. H., Saenz D., Baum K. A., Langerhans R. B. & DeWitt T. J. (2005) Body shape, burst speed and escape behavior in larval anurans. *Oikos*, **111**, 582-591.

De Block M. & Stoks R. (2008a) Short-term larval food stress and associated compensatory growth reduce adult immune function in a damselfly. *Ecological Entomology*, **33**, 796-801.

De Block M. & Stoks R. (2008b) Compensatory growth and oxidative stress in a damselfly. *Proceedings of the Royal Society London B*, **275**, 781-785.

Decaestecker E., De Meester L. & Ebert D. (2002) In deep trouble: habitat selection constrained by multiple enemies in zooplankton. *PNAS*, **99**, 5481-5485.

Dmitriew C. (2011) The evolution of growth trajectories: what limits growth rate? *Biological Reviews*, **86**, 97-116.

399 Edeline E., Ben Ari T., Vollestad L. A., Winfield I. J., Fletcher J. M., Ben James J. *et al.*
400 (2008) Antagonistic selection from predators and pathogens alters food-web structure.
401 *PNAS*, **105**, 19792-19796.
402 Espinoza-Urgel M. & Kolter R. (1998) *Escherichia coli* genes expressed preferentially in an
403 aquatic environment. *Molecular Microbiology*, **28**, 325-332.
404 Fox G.A. (2001) Failure time-analysis: Studying times to event and rates at which events
405 occur. In: *Design and analysis of ecological experiments* (eds S. M. Scheiner & J.
406 Gurevitch), pp.235-266, Oxford University Press, Oxford.
407 Freitak D., Wheat C. W., Heckel D. G. & Vogel H. (2007) Immune system responses and
408 fitness costs associated with consumption of bacteria on larvae of *Trichoplusia ni*. *BMC*
409 *Biology*, **5**, 56.
410 Freitak D., Heckel D. G. & Vogel H. (2009) Bacterial feeding induces changes in immune-
411 related gene expression and has trans-generational impacts in the cabbage looper
412 (*Trichoplusia nii*). *Frontiers in Zoology*, **6**, 10.1186/1742-9994-6-7.
413 Gonzalez-Santoyo I. & Cordoba-Aguilar A. (2012) Phenoloxidase: a key component of the
414 insect immune system. *Entomologia Experimentalis et Applicata*, **142**, 1-16.
415 Guerrero-Beltran J. A. & Barbosa-Canovas G. V. (2005) Reduction of *Saccharomyces*
416 *cerevisiae, Escherichia coli* and *Listeria innocua* in apple juice by ultraviolet light. *Journal*
417 *of Food Process Engineering*, **28**, 437-452.
418 Hatcher M. J., Dick J. T. A. & Dunn A. M. (2006) How parasites affect interactions between
419 competitors and predators. *Ecology Letters*, **9**, 1253-1271.
420 Jacot A., Scheuber H. & Brinkhof M. W. G. (2004) Costs of an induced immune response on
421 sexual display and longevity in field crickets. *Evolution*, **58**, 2280-2286.

Janssens L. & Stoks R. (2012) How does a pesticide pulse increase vulnerability to predation? Combined effects on behavioral traits and escape swimming. *Aquatic Toxicology*, **110-111**, 91-98.

Janssens L. & Stoks R. (2013) Exposure to a widespread non-pathogenic bacterium magnifies sublethal pesticide effects in the damselfly *Enallagma cyathigerum*: from the suborganismal level to fitness-related traits. *Environmental Pollution,* **177**, 143-149.

Johansson H., Stoks R., Nilsson-Örtman V., Ingvarsson P.K. & Johannson F. (2013) Large-scale patterns in genetic variation, gene flow and differentiation in five species of European Coenagrionid damselfly provide mixed support for the central-marginal hypothesis. *Ecography*, **36**, 744-755.

Kirschner A.K.T. & Velimirov B. (1997) A seasonal study of bacterial community succession in a temperate backwater system, indicated by variation in morphotype numbers, biomass, and secondary production. *Microbial Ecology*, **34**, 27-38.

Kulkoyluoglu O., Dugel M. & Kilic M. (2007) Ecological requirements of Ostracoda (Crustacea) in a heavily polluted shallow lake, Lake Yenicaga (Bolu, Turkey). *Hydrobiologia*, **585**, 119-133.

Leff L. G. & Lemke M. J. (1998) Ecology of aquatic bacterial populations: lessons from applied microbiology. *Journal of the North American Benthological Society*, **17**, 261-271.

McPeek M. A., Schrot A. K. & Brown J. M. (1996) Adaptation to predators in a new community: Swimming performance and predator avoidance in damselflies. *Ecology*, **77**, 617-629.

Moret Y. & Schmid-Hempel P. (2000) Survival for immunity: the price of immune system activation for bumblebee workers. *Science*, **290**, 1166-1168.

Mortensen L. & Richardson J. M. L. (2008) Effects of chemical cues on foraging in damselfly larvae, *Enallagma antennatum*. *Journal of Insect Behavior*, **21**, 285-295.

447 Nagel L., Mlynarek J. J. & Forbes M. R. (2011) Immune response to nylon filaments in two
448 damselfly species that differ in their resistance to ectoparasitic mites. *Ecological*
449 *Entomology*, **36**, 736-743.
450 Otti O., Gantenbein-Ritter I., Jacot A. & Brinkhof M. W. (2012) Immune response increases
451 predation risk. *Evolution*, **66**, 732-739.
452 Palijan G. (2012) Abundance and biomass responses of microbial food web components to
453 hydrology and environmental gradients within a floodplain of the river Danube. *Microbial*
454 *Ecology*, **64**, 39-53.
455 Perrot-Minnot M. J., Maddaleno M., Balourdet A. & Cézilly F. (2012) Host manipulation
456 revisited: no evidence for a causal link between altered photophobia and increased trophic
457 transmission of amphipods infected with acanthocephalans. *Functional Ecology*, **26**, 1007-
458 1014.
459 Rantala M. J., Honkavaara J. & Suhonen J. (2010) Immune system activation interacts with
460 territory-holding potential and increases predation of the damselfly *Calopteryx splendens*
461 by birds. *Oecologia*, **163**, 825-832.
462 Rolff J. & Siva-Jothy M. T. (2003) Invertebrate ecological immunology. *Science*, **301**, 472-
463 475.
464 Roth O. & Kurtz J. (2008) The stimulation of immune defence accelerates development in the
465 red flour beetle (*Tribolium castaneum*). *Journal of Evolutionary Biology*, **21**, 1703-1710.
466 Schmid-Hempel P. (2005) Evolutionary ecology of insect immune defenses. *Annual Review*
467 *of Entomology*, **50**, 529-551.
468 Sheldon B. C. & Verhulst S. (1996) Ecological immunology: costly parasite defences and
469 trade-offs in evolutionary ecology. *Trends in Ecology and Evolution*, **11**, 317-321.
470 Siva-Jothy M. T., Moret Y. & Rolff J. (2005) Insect immunity: an evolutionary ecology
471 perspective. *Advances in Insect Physiology*, **32**, 1-48.

472 Siva-Jothy M. T. & Thompson J. J. W. (2002) Short-term nutrient deprivation affects immune
473 function. *Physiological Entomology*, **27**, 206-212.
474 Stoks R. & De Block M. (2011) Rapid growth reduces cold resistance: evidence from
475 latitudinal variation in growth rate, cold resistance and stress proteins. *PLoS ONE*, **6**,
476 e16935.
477 Stoks R., De Block M. & McPeek M. A. (2005a) Alternative growth and energy storage
478 responses to mortality threats in damselflies. *Ecology Letters*, **8**, 1307-1316.
479 Stoks R., De Block M., Slos S., Van Doorslaer W. & Rolff J. (2006) Time constraints mediate
480 predator-induced plasticity in immune function, condition and life history. *Ecology*, **87**,
481 809-815.
482 Stoks R., De Block M., Van de Meutter F. & Johansson F. (2005b) Predation cost of rapid
483 growth: behavioural coupling and physiological decoupling. *Journal of Animal Ecology*,
484 **74**, 708-715.
485 Stoks R. & McPeek M. A. (2006) A tale of two diversifications: Reciprocal habitat shifts to
486 fill ecological space along the pond permanence gradient. *American Naturalist*, **168**, S50-
487 S72.
488 Stoks R., McPeek M. A. & Mitchell J. L. (2003) Evolution of prey behavior in response to
489 changes in predation regime: Damselflies in fish and dragonfly lakes. *Evolution*, **57**, 574-
490 585.
491 Stoks R., Nystrom J. L., May M. L. & McPeek M. A. (2005c) Parallel evolution in ecological
492 and reproductive traits to produce cryptic damselfly species across the holarctic. *Evolution*,
493 **59**, 1976-1988.
494 Strobbe F., McPeek M. A., De Block M., De Meester L. & Stoks R. (2009) Survival selection
495 on escape performance and its underlying phenotypic traits: a case of many-to-one
496 mapping. *Journal of Evolutionary Biology*, **22**, 1172-1182.

497 Valtonen T. M., Kleino A., Ramet M. & Rantala M. J. (2010) Starvation reveals maintenance
498 cost of humoral immunity. *Evolutionary Biology*, **37**, 49-57.
499 Van Noordwijk A. J. & de Jong G. (1986) Acquisition and allocation of resources: their
500 influence on variation in life history tactics. *American Naturalist*, **128**, 137-142.
501 Willer Y., Müller B. & Bumann D. (2012) Intestinal inflammation responds to microbial
502 tissue load independent of pathogen/non-pathogen discrimination. *PLOS ONE*, **7**, e35992.
503 Yin M., Laforsch C., Lohr J. N. & Wolinska J. (2011) Predator-induced defense makes
504 *Daphia* more vulnerable to parasites. *Evolution*, **65**, 1482-1488.
505

Table 1. Results of the repeated-measures ANCOVAs testing for the effects of previous bacterial exposure and predation risk on activity-related variables in the damselfly *Enallagma cyathigerum.* Mass was included as a covariate. The successive observations of an activity in the absence and in the presence of predator cues were considered repeats with predation risk being the within-subject factor.

		df_1, df_2	*F*	*P*
Walks	Predation Risk (Pr)	1, 57	2.86	0.097
	Bacterium (Ba)	1, 57	30.52	< 0.0001
	Pr × Ba	1, 57	6.77	0.012
	Mass	1, 57	4.50	0.038
Feeding strikes	Predation risk (Pr)	1, 57	0.72	0.40
	Bacterium (Ba)	1, 57	52.09	< 0.0001
	Pr × Ba	1, 57	24.01	< 0.001
	Mass	1, 57	3.69	0.059

520 **Figure legends**

521 Figure 1. Effects of exposure to a non-pathogenic strain of the bacterium *E. coli* on (a) the
522 growth rate, and two components of immune defence, (b) activity of phenoloxidase (PO) and
523 (c) the number of haemocytes of *E. cyathigerum* larvae. Given are least-squares means with
524 95% confidence intervals. Phenoloxidase activity was corrected for protein concentration.
525

526 Figure 2. Effects of previous exposure to a non-pathogenic strain of the bacterium *E. coli* and
527 predation risk on activity-related variables of *E. cyathigerum* larvae: (a) number of walks and
528 (b) feeding strikes. Least-squares means with 95% confidence intervals are shown.

529

530 Figure 3. Effects of previous exposure to a non-pathogenic strain of the bacterium *E. coli* on
531 variables related with escape swimming of *E. cyathigerum* larvae: (a) swimming propensity
532 and (b) swimming speed. Least-squares means with 95% confidence intervals are shown.

533

534 Figure 4. Kaplan-Meier survival curves of *E. cyathigerum* larvae when exposed to an *Anax*
535 dragonfly predator as a function of previous exposure to the bacterium *E. coli*. Larvae alive
536 after 120 minutes were considered right-censored.

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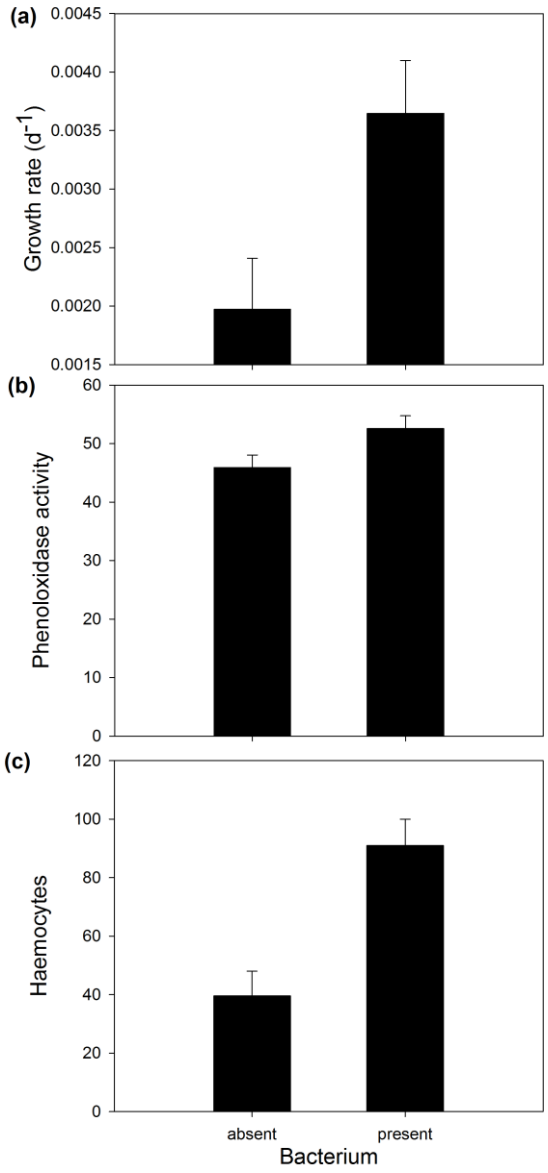
541 Figure 1



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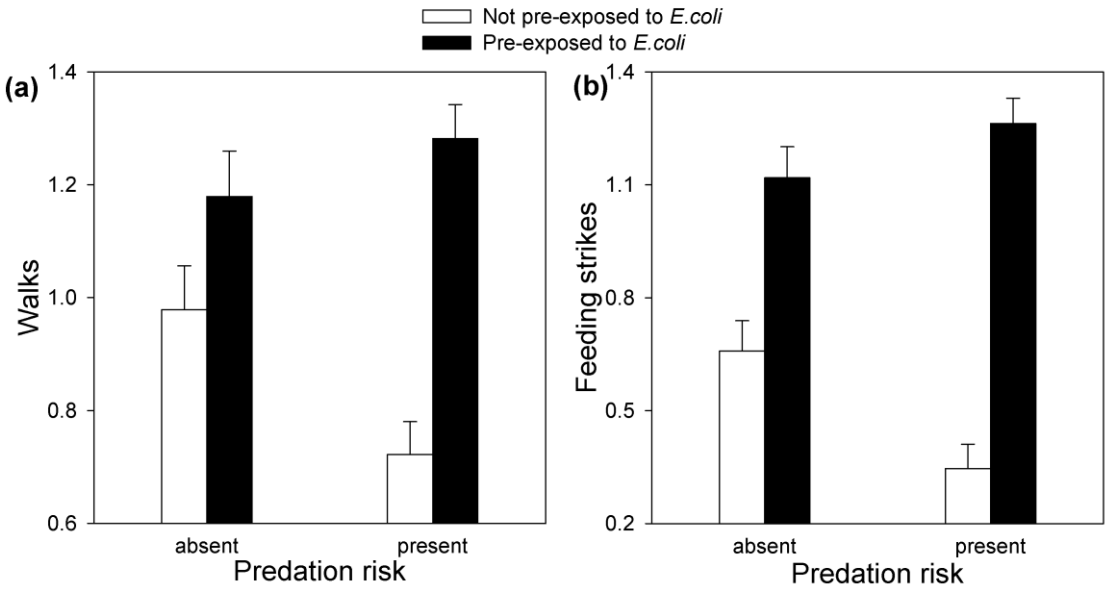
544 Figure 2



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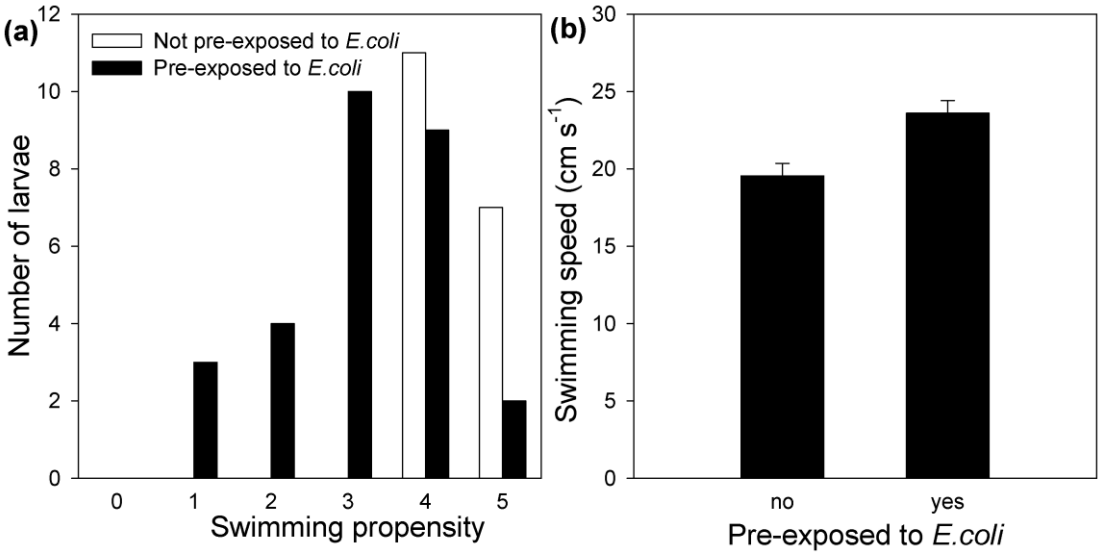
547 Figure 3



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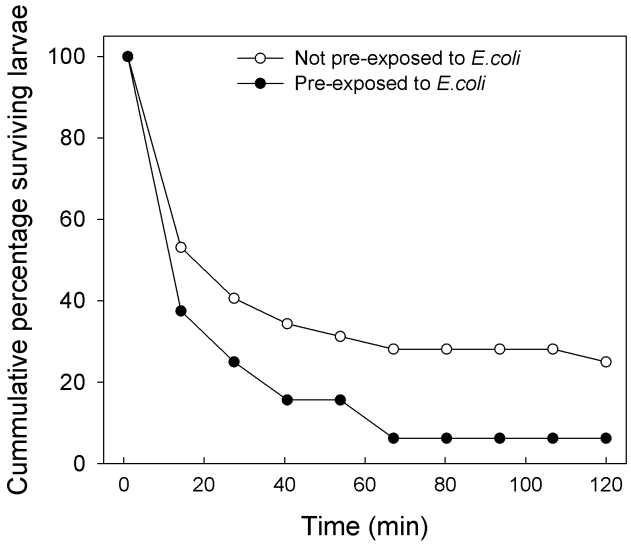
550 Figure 4



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